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Factors affecting the amount and the mode of merocyanine 540 binding to the membrane of human erythrocytes. A comparison with the binding to leukemia cells

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Abstract

In the presence of albumin Merocyanine 540 (MC540) exhibits a very limited binding to the outer surface of the membrane of normal erythrocytes, whereas pronounced binding is observed to leukemia cells. To find out whether this difference is due to differences in the composition or structural organization of the cell membrane we analyzed effects of a number of covalent and non-covalent perturbations of the red cell membrane on the binding and fluorescence characteristics of membrane-bound MC540. It is shown that exposure of the cells to cationic chlorpromazine, neuraminidase or photodynamic treatment with $AlPcS_4$ as sensitizer caused a limited increase (30–50%) of MC540 binding, together with a red shift of the fluorescence emission maximum and an increase of the relative fluorescence quantum yield of membrane-bound MC540. Other forms of perturbation of the membrane structure, like hyperthermia (48° C) and treatments that produce a decrease of phospholipid asymmetry in addition to accelerated flip-flop, did not result in increased MC540 binding, but did cause a red shift of the fluorescence emission maximum and an increase of the relative fluorescence quantum yield. These changes in fluorescence properties indicate a penetration of the dye into more hydrophobic regions in the membrane. MC540, bound to Brown Norway myelocytic leukemia cells, exhibited a red shift of the fluorescence emission maximum and an increased relative fluorescence quantum yield as compared to MC540 bound to untreated erythrocytes. These changes were of the same order of magnitude as in photodynamically treated red blood cells. Dye binding per surface area, however, was about 3-times higher with these leukemia cells than with photodynamically treated red blood cells. This demonstrates that certain perturbations of the erythrocyte membrane evoked a MC540 binding that became qualitatively comparable to the dye binding to leukemia cells, although dye binding per surface area was still significantly lower.

Keywords: Merocyanine 540; Erythrocyte membrane; Leukemia cell

1. Introduction

Merocyanine 540 is a heterocyclic chromophore with a localized negative charge, that does not readily penetrate intact cells. Binding to intact cells is therefore initially limited to the outer leaflet of the lipid bilayer of the plasma membrane [1]. Many recent studies address the possible use of MC540 as photosensitizer for bone marrow purging in the treatment of leukemia [1–4]. This is based on the observation that MC540 effectively sensitizes the photodynamic killing of leukemia cells, whereas normal bone marrow and blood cells are much less affected. It appeared that the different sensitivities of leukemia and normal cells can, at least partly, be ascribed to the much higher binding of the sensitizer to leukemia cells ('high-affinity binding') compared to normal cells ('low-affinity binding') [5,6]. That means that the therapeutic efficiency of MC540-sensitized photodynamic bone marrow purging

Abbreviations: $AlPcS_4$, aluminum chlorotetrasulfophthalocyanine; BNML cells, Brown Norway myelocytic leukemia cells; BSA, bovine serum albumin; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; MC540, Merocyanine 540; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); *t*-BuOOH, *t*-butylhydroperoxide; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

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might be increased by manipulating the binding of MC540 to normal and/or malignant cells. In this context it is important to clarify the molecular and structural background of low-affinity binding of MC540 to normal cells, versus high-affinity binding to leukemia cells.

Considering the MC540 binding to the outer membrane leaflet, the difference between high- and low-affinity binding must reflect differences in composition and/or structural organization of the membrane. Based on experiments with liposomes it was shown that MC540 binds preferentially to cholesterol-free phospholipid domains in membranes and to bilayers with relatively loose fatty acid packing [7–10]. In accordance it has been suggested that a decrease of the normal membrane phospholipid asymmetry in erythrocytes and lymphocytes led to increased MC540 binding [11–14], as loss of asymmetry leads to a less tight packing of the outer leaflet of the membrane.

Experiments reported by Allan et al. [15] did not support this assumption, however. These authors prepared human erythrocyte ghosts that had maintained their normal phospholipid asymmetry but were permeable to MC540. It appeared that these asymmetric, open ghosts bound only twice as much MC540 as compared to intact erythrocytes. Apparently the inner leaflet, with its high concentration of phosphatidylethanolamine and phosphatidylserine, which are rich in unsaturated, loosely packed fatty acids, did not bind more MC540 than the more closely packed outer leaflet, contradicting earlier suggestions in the literature.

When comparing experimental results reported by different laboratories, a complicating factor is the use of different methods for measuring MC540 binding to the cells. In some cases bound MC540 was extracted from the cells with subsequent measurement of the dye concentration in the extraction fluid. In other studies binding was assayed by measuring directly the fluorescence of MC540-loaded cells. A direct comparison of results obtained by these two different approaches is hampered by the fact that the fluorescence yield of bound MC540 not only depends on the amount of dye actually bound to the cells, but also on other factors like membrane potential and orientation of the dye molecules with respect to the phospholipid molecules, e.g., parallel or perpendicular [16,17].

Therefore, a systematic study of the effects of perturbation of the erythrocyte membrane structure with respect to bilayer asymmetry, lipid packing, surface charge and some other parameters on the amount and mode of MC540 binding was undertaken and the results compared with the binding to leukemia cells, in order to try to explain the difference between the low-affinity binding in erythrocytes and the high-affinity binding in leukemia cells.

2. Materials and methods

MC540 was purchased from Eastman Kodak, Rochester, NY. Stock solutions (1 mg/ml) were prepared in 50% ethanol. Chlorpromazine was obtained from Specia, Paris, $AlPcS_4$ from Eastman Kodak, Rochester, NY, DIDS from Pierce, Rockford, IL, TMA-DPH from Molecular Probes, Eugene, OR, Dextran 4 from Serva, Heidelberg, Germany and tissue culture products from Gibco-BRL, Breda, The Netherlands. All other chemicals were from Sigma, St. Louis, MO, USA or Baker, Deventer, The Netherlands.

Heparinized human blood was centrifuged shortly after collection. The erythrocytes were washed three times in PBS and, unless otherwise specified, resuspended at 2% hematocrit in a medium containing (mM) KCl, 90; NaCl, 45; Na_2HPO_4/NaH_2PO_4, 12.5, pH 7.4 and sucrose, 44 (medium A). Open erythrocyte ghosts were prepared as described by Weed et al. [18]. BNML cells [19] were cultured in RPMI 1640, supplemented with 10% fetal calf serum and 4 mM glutamine.

Binding of MC540 to erythrocytes and ghosts was assayed as described by Allan et al. with some modifications [15]. Briefly, a 1 ml erythrocyte suspension was incubated in the dark with 17.5 μM MC540 in the presence of 0.15% BSA at 22° C. BSA was added to eliminate nonspecific binding of MC540 by competing for dye molecules [20]. After centrifugation in an Eppendorf centrifuge (14 000 rpm for 5 min) the supernatant was removed. The pellet was resuspended in 1 ml medium A and a 100 μl sample of this suspension was added to 2 ml of n-butanol and vortexed for 10 s. The amount of MC540 in the butanol solution was determined by measuring the fluorescence (excitation 540 nm, emission 580 nm) with an Aminco SPF-500 spectrofluorometer. In experiments in which the relative affinity of MC540 for the erythrocyte membrane under varying experimental conditions was estimated, the release of cell-bound MC540 during two washing steps of the cells in 1 ml medium A containing 0.15% BSA and one washing step in 1 ml medium A was monitored.

To measure the relative fluorescence quantum yield and emission maximum of cell-bound MC540, cells and medium were separated by centrifugation. The cells were resuspended in fresh medium A at a hematocrit of 0.02% and immediately assayed for fluorescence (excitation 540 nm). A second sample was treated identically but centrifuged immediately after resuspending. The fluorescence of the supernatant, representing MC540 released from the cells, was subtracted from the fluorescence of the cell suspension, to obtain fluorescence data of cell-bound MC540. The so obtained spectrum of cell-bound MC540 was not affected by addition of hemoglobin to the supernatant in a concentration comparable to a 0.02% erythrocyte suspension. The relative fluorescence quantum yield is defined as the quantum yield of cell-bound MC540 divided by the quantum yield of the dye at the same concentration in aqueous solution, both measured at the emission maximum [21].

In parallel experiments the relative fluorescence quantum yield of cell-bound MC540 was assayed by flow

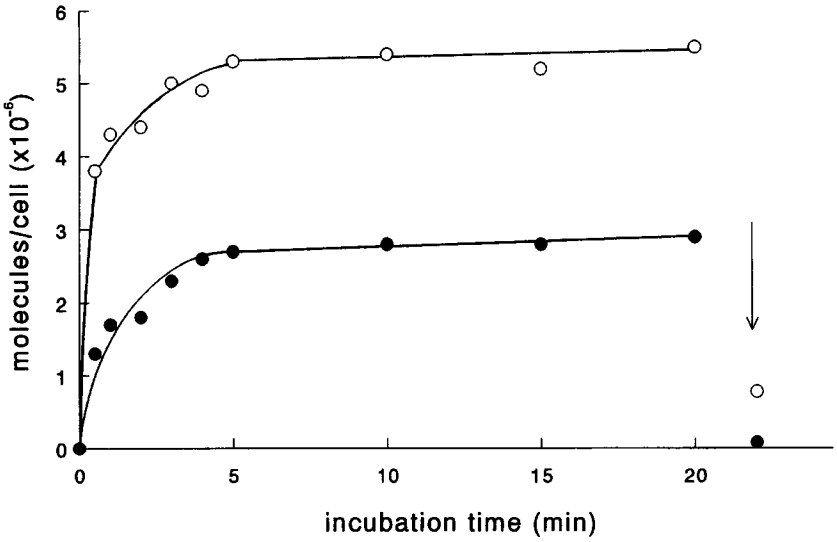

Fig. 1. Binding of MC540 to human erythrocytes (●) and open ghosts (○), in the presence of 0.15% BSA in the medium. Initial MC540 concentration in the medium: 17.5 μM. Arrow: cells subjected to three washing steps with albumin-containing buffer.

cytometry in a Becton-Dickinson FACS-IV fluorescence activated cell sorter (exc. 514 nm, em. > 525 nm). The results with these two methods were always in good agreement.

Treatment of erythrocytes with tetrathionate or diamide was done as described by Haest et al. [22]. Briefly, an erythrocyte suspension (hematocrit 10%) was treated with 20 mM tetrathionate or with 5 mM diamide for 40 min at 37° C, pH 8.0, followed by a further incubation in fresh medium A. Lowering of the cytoplasmic ATP concentration was performed as described by Middelkoop et al. [23]. Cytoplasmic ATP concentrations were measured with firefly lantern extract, using a BioOrbit 1251 Luminometer. Pre-incubation of erythrocytes with 5 μM gramicidin was performed at 37° C for 30 min [24] and with 100 μM chlorpromazine or 100 μM decanol at 22° C during 30 min. Erythrocytes were incubated with 2 mM *t*-BuOOH as described by Classen et al. [25], with 10% PEG 6000 as described by Schwichtenhovel et al. [26] and with DIDS according to Lepke et al. [27]. Illumination of MC540-loaded cells with white light was done as described before [28]. Photodynamic treatment of erythrocytes with $AlPcS_4$ (2.5 μM) as sensitizer was performed as described previously [29]. Enzymatic removal of sialic acid residues from the outer surface of the erythrocyte membrane was accomplished as described by Goubran Botros et al. [30]. Electroporation was carried out as described by Dressler et al. [31]. Briefly, an erythrocyte suspension (30% hematocrit)

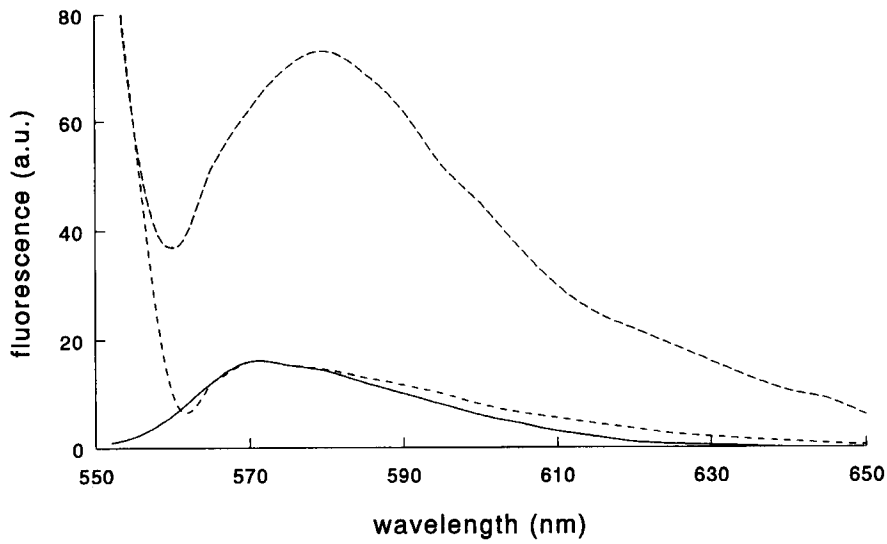

Fig. 2. Fluorescence emission spectra (excitation 540 nm) of unbound MC540 in medium A (—) erythrocyte-bound MC540 (- - - -) and MC540 bound to open ghosts (— —). The final MC540 concentration was 1 μM in all cases.

in medium A containing 8.8% (w/v) Dextran 4 instead of sucrose was filled in a discharge chamber at 0° C. The suspension was exposed to 80 μs field pulses at 8 kV/cm. To reseal the permeability barrier the suspension was incubated for 1 h at 37° C, before exposing the cells to MC540.

The cholesterol content of erythrocyte membranes was lowered as described by Gottlieb [32]. Briefly, cell-free human plasma was incubated at 37° C. After 72 h erythrocytes (10% hematocrit) and glucose (0.25%) were added and the incubation was continued for another 16 h. Approx. 35% of the cholesterol was removed from the membranes by this procedure. Cholesterol was extracted as described by Rose and Oklander [33] and assayed as described by Omodeo Salè et al. [34]. Lipid peroxidation was essayed by measuring the generation of thiobarbituric acid reactive species as described by Miller et al. [35] and the formation of fatty acyl hydroperoxides as described by Thomas and Girotti [36].

Fluorescence microscopy was performed with a Bio-Rad MRC 600 confocal laserscan microscope (exc. 488 nm, em. > 510 nm).

All measurements were done at least eight times with an experimental error of < 8%.

3. Results

3.1. Characterization of binding of MC540 to intact erythrocytes and ghosts

The binding of MC540 in the presence of 0.15% BSA to intact erythrocytes and to open ghosts, as measured after extraction of bound dye with n-butanol, is depicted in Fig. 1. The binding to intact erythrocytes levels off after about 5 min at a binding of $2.7 \cdot 10^6$ molecules/cell. The emission maximum of erythrocyte-bound MC540 was at 572 nm and the fluorescence yield was equal to the yield of unbound MC540 in medium A at the same concentration (Fig. 2). After washing the cells with albumin-containing buffer as described in the methods section, dye binding decreased by 97% to $0.08 \cdot 10^6$ molecules/cell (Fig. 1). The same results were obtained at 0° C. Treatment of the cells with DIDS, which blocks the anion binding site of band 3, caused a slightly reduced MC540 binding, without affecting the other binding characteristics (Table 1). MC540 binding to open ghosts amounted to $5.4 \cdot 10^6$ molecules/cell (Fig. 1). The fluorescence emission maximum was red-shifted to 582 nm and the relative fluorescence quantum yield was increased to 4.7 (Fig. 2). Washing of the ghosts with albumin-containing buffer released about 85% of the initially bound MC540 (Fig. 1).

3.2. Effect of treatments that enhance transbilayer reorientation of lipids

Gramicidin enhances the transbilayer reorientation (flip-flop) of lipids in the erythrocyte membrane, without affecting the normal phospholipid asymmetry between the inner and outer leaflet [24]. As shown in Table 1 treatment of erythrocytes with gramicidin had no effect on any of the MC540 binding characteristics. Treatment of the cells with *t*-BuOOH, also known to enhance the flip-flop rate about 15-fold at a concentration of 2 mM [25] again failed to affect the binding characteristics (Table 1).

Treatment of intact red blood cells with SH-oxidizing

Table 1
The effects of perturbation of the structure of the erythrocyte membrane on the characteristics of MC540 binding

Pretreatment	Binding, molecules/cell ($\times 10^{-6}$)	Em. max., nm	Rel. fluoresc. quantum yield	Washed out, %	Binding after washing, molecules/cell ($\times 10^{-6}$)
None	2.7	572	1.0	97	0.08
Ghosts	5.4	582	4.7	85	0.80
DIDS	2.5	572	1.1	98	0.05
Gramicidin	2.7	572	0.99	96	0.10
t-BuOOH	2.8	572	0.97	97	0.08
Tetrathionate	2.7	572	0.93	96	0.10
Tetrathionate + ATP depletion	2.4	585	6.5	88	0.29
Diamide	2.8	572	1.1	97	0.08
Diamide + ATP depletion	2.5	585	4.7	84	0.40
Electroporation	3.1	582	4.4	84	0.26
Cholesterol depletion	2.6	572	1.0	97	0.08
$AlPcS_4$ + light, 20 min	3.5	585	7.8	87	0.44
Light (MC540), 5 min	2.6	585	5.6	83	0.17
Heat, 46° C, 2 h	2.8	572	1.0	97	0.08
Heat, 48° C, 2 h	2.6	585	5.8	96	0.09
Neuraminidase	3.4	580	2.7	98	0.09
PEG, 10%	2.7	575	2.2	96	0.10
Decanol	2.6	580	2.5	97	0.08
Chlorpromazine	4.1	585	6.3	79	0.84
Chlorpromazine, 0° C	3.6	585	6.2	92	0.28

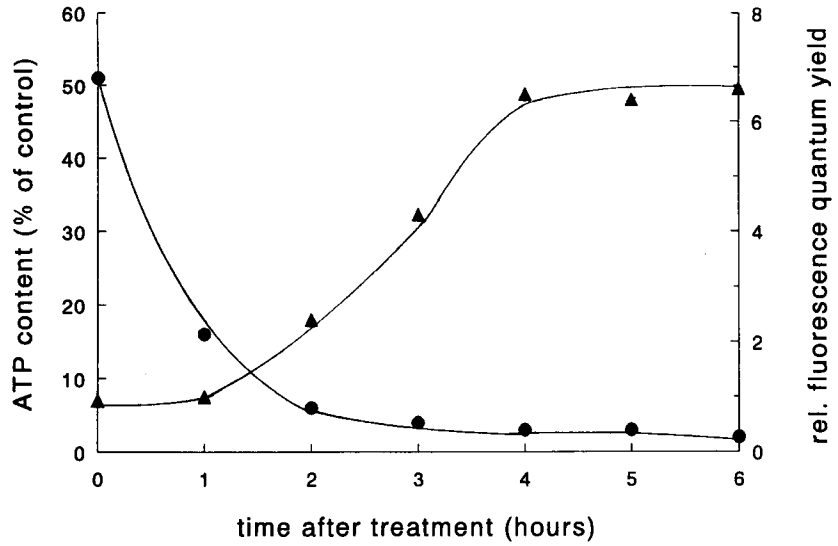

Fig. 3. The effect of incubation after tetrathionate treatment on cytosolic ATP concentration (●) and relative fluorescence quantum yield of cell-bound MC540 (▲).

reagents like diamide or tetrathionate did not lead to a change of MC540 binding characteristics, when measured immediately after treatment. During further incubation of the cells in reagent-free buffer A, however, a gradual shift of the emission maximum to 585 nm and a pronounced increase of the relative fluorescence quantum yield could be observed, without increased MC540 binding to the cells (Fig. 3, Table 1). These changes started when the ATP content of the cytosol had decreased to about 6% of the original value. In further experiments it could be demonstrated that the decreased ATP concentration was crucial for the observed changes of the fluorescence characteristics of bound MC540. If the erythrocytes were ATP-depleted before treatment with tetrathionate, the increased fluorescence quantum yield and the red-shift of the emission maximum occurred immediately after treatment (not shown). These results indicate that loss of phospholipid asymmetry is involved in the changed fluorescence characteristics of bound MC540 [23]. In line with these findings a decrease of phospholipid asymmetry produced by electroporation of cells followed by resealing [31] also resulted in a red-shift of the emission maximum and an increase of the fluorescence quantum yield (Table 1). The amount of MC540 that remained bound to the cells after the standard washing procedure was substantially higher (12–16%) than with control cells (3.1%, Table 1).

3.3. Effect of cholesterol depletion

Cholesterol depletion of the membrane may lead to a less tight packing of the phospholipids in the outer leaflet of the membrane [8]. However, extraction of about 35% of

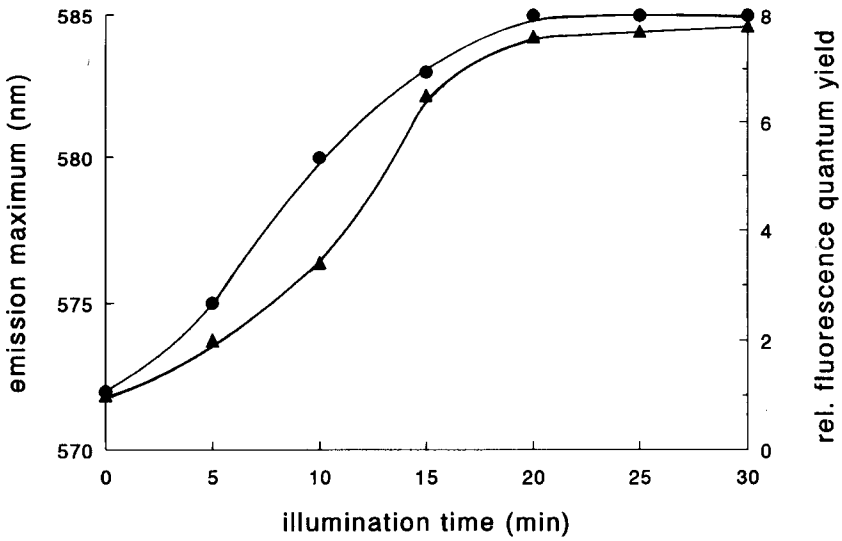

Fig. 4. The effect of photodynamic treatment with $AlPcS_4$ as sensitizer on emission maximum (●) and relative fluorescence quantum yield (▲) of cell-bound MC540.

Table 2
Characteristics of MC540 binding to normal, intact erythrocytes, $AlPcS_4$-photodynamically treated erythrocytes and to BNML cells

	Normal erythrocytes	$AlPcS_4$-photodynamically treated erythrocytes	BNML cells
Binding, molecules/cell ($\times 10^{-6}$)	2.7	3.5	100
Cholesterol content, fmol/cell	0.34	0.34	3.6
Surface area, μm^2/cell	135	135	1286
Binding, molecules/μm^2 ($\times 10^{-3}$)	20	26	78
Em. max, nm	572	585	580
Rel. fluoresc. quantum yield	1.0	7.8	5.6
Washing out, %	97	87	84
Binding after washing, molecules/cell ($\times 10^{-6}$)	0.08	0.44	16
Binding after washing, molecules/μm^2 ($\times 10^{-3}$)	0.59	3.38	12

the cholesterol from the membrane, changing the cholesterol/phospholipid ratio (w/w) from 0.40 to 0.23, had no effect on the MC540 binding characteristics (Table 1).

3.4. Effect of photodynamic treatment

Treatment of red blood cells with the sensitizer $AlPcS_4$ in the dark did not affect MC540 binding. If these cells were illuminated during 20 min, however, subsequent incubation with MC540 led to a 30% increase of MC540 binding, a shift of the emission maximum to 585 nm, an eightfold increase of the relative fluorescence quantum yield and a decreased extraction of the dye during subsequent washing (Fig. 4, Table 1). As MC540 itself is a photosensitizer, similar experiments were conducted with cells, incubated with MC540 only. During illumination of the cells MC540 binding did not increase, but the emission maximum shifted to 585 nm, again with a concomitant increase of the relative fluorescence quantum yield and a decreased extraction of dye by washing (Table 1). These photodynamic treatments of the erythrocytes did not cause measurable lipid peroxidation as judged from the lack of generation of thiobarbituric acid reactive products and fatty acyl hydroperoxides (not shown).

3.5. Effect of various other membrane structure-perturbing treatments

The effects of various other treatments leading to perturbation of the membrane structure are summarized in Table 1. Heat treatment at 48° C (but not at 46° C) for 2 h caused a red-shift of the fluorescence emission peak and an increased fluorescence quantum yield. This effect was observed when the red cells were heat-treated and subsequently cooled to room temperature before MC540 was added. Pretreatment of the cells with neuraminidase or

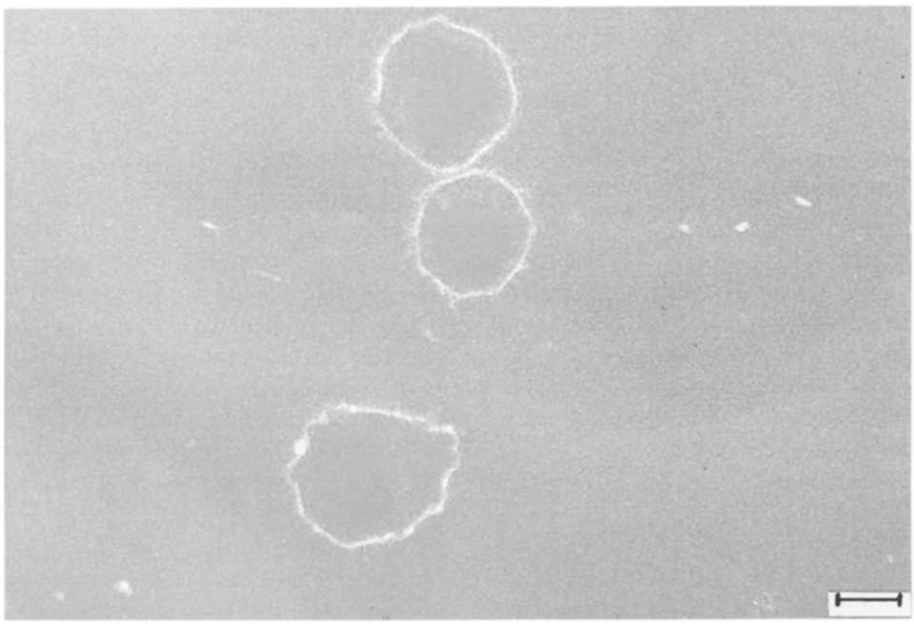

Fig. 5. Fluorescence micrograph of BNML cells incubated with 17.5 μM MC540 in the presence of 0.15% BSA. Original magnification: 800 ×. Bar = 10 μm.

addition of PEG 6000 or decanol to the incubation medium had a similar effect, although less pronounced. Finally the effect of chlorpromazine, known to intercalate between the phospholipid molecules in the membrane, was investigated. As shown in Table 1 MC540 binding increased by 50%, the emission maximum of cell-bound dye shifted to 585 nm and the relative fluorescence quantum yield increased 6-fold. Because chlorpromazine induces endocytosis [37] MC540 binding to chlorpromazine-treated cells was also measured at 0° C. Binding was slightly lower at 0° C whereas the amount of MC540 that remained bound to the cells after the standard washing procedure was substantially lower (8%) at 0° C than at room temperature (21%).

3.6. MC540 binding to BNML cells

Table 2 summarizes the binding characteristics of MC540 to BNML cells, as compared to human erythrocytes. Also in this case the same results were obtained at 0° C. Fluorescence microscopy showed that the binding was restricted to the plasma membrane (Fig. 5). The binding in molecules/cell was recalculated to binding per surface area. Human erythrocytes have a mean surface area of 135 μm^2 [38] and a cholesterol content of 0.34 fmol/cell. Lange et al. showed that in many cells the ratio cholesterol content/surface area is the same [39]. The cholesterol content of BNML cells was 3.6 fmol/cell. The surface area calculated in this way amounted to 1286 μm^2. Comparable results were obtained when measuring the surface area of BNML cells with the fluorescent dye TMA-DPH as described by Bronner et al. [40].

The emission maximum of cell-bound MC540 was red-shifted compared to MC540 bound to red blood cells (580 nm versus 572 nm) and the relative fluorescence quantum yield was 5.6-times higher. The percentage of MC540 that remained bound to the cells after washing with albumin-containing buffer was much higher in BNML cells, compared to erythrocytes (Table 2).

4. Discussion

Under the experimental conditions used in this study, MC540 binding to intact human erythrocytes amounted to $2.7 \cdot 10^6$ molecules/cell. It is highly unlikely that this binding is caused by endocytosis, as changing the temperature from 22° C to 0° C had no effect. Binding to the anion binding site of band 3 molecule could also be excluded. After treatment of the cells with DIDS, MC540 binding was only slightly reduced (Table 1).

The fluorescence emission maximum of MC540 bound to untreated erythrocytes was at 572 nm. The fluorescence emission maximum of MC540 depends on the dielectric constant of the environment, changing from about 572 nm in buffer, to about 585 nm in solvents with a low dielectric constant [41]. This red-shift is attended with an 8- to 12-fold increase of the relative fluorescence quantum yield [21]. In model experiments it has been shown that MC540 binding to phospholipid layers can occur in at least two ways [10,16,17]. The first possibility is that the dye is largely in contact with the water phase with its long axis perpendicular to the long axis of the phospholipid molecules. In this case the fluorescence emission maximum is at 572 nm [10]. Secondly, the dye is predominantly in contact with the hydrocarbon matrix with its long axis parallel to the long axis of the phospholipid molecules. In this configuration MC540 exhibits a fluorescence emission maximum at 585 nm [10]. Based on these observations, it seems highly likely that in untreated erythrocytes most bound MC540 molecules are in close contact with the surrounding water with their long axis perpendicular to the long axis of the phospholipids.

Several studies emphasize the importance of lipid packing in the outer leaflet of the membrane bilayer for MC540 binding [11–14]. Normally the phospholipids in the erythrocyte membrane are distributed asymmetrically with most of the phosphatidylcholine and sphingomyelin localized in the outer leaflet and most of the phosphatidylserine and -ethanolamine in the inner leaflet [42]. This asymmetry is maintained by an ATP-dependent aminophospholipid translocase ('flippase') [43,44], together with an interaction of spectrin with the anionic phospholipids in the inner leaflet of the bilayer [45]. Because the fatty acid chains of phosphatidylcholine and sphingomyelin are much more saturated than the acyl chains of phosphatidylserine and -ethanolamine, the outer leaflet is much more tightly packed than the inner leaflet. Thus, if lipid packing is indeed important in MC540 binding, it should be expected that leaky ghosts would bind appreciably more than twice the amount of MC540, compared to intact cells. However, as also shown by Allan et al. [15], binding to open ghosts is only twice the amount bound to intact cells (Fig. 1, Table 1). It should also be expected that erythrocytes that had lost their normal phospholipid asymmetry, causing a less tight lipid packing in the outer leaflet, bind more MC540 than untreated cells. However treatment of cells with SH-oxidizing agents combined with inhibition of the flippase by ATP-depletion [23], or electroporation of cells [31] did not enhance the amount of bound MC540. But in all these cases the mode of MC540 binding changed. The emission maximum of bound MC540 was red-shifted to 582–585 nm, compared to 572 nm for MC540 bound to untreated erythrocytes. Also the relative fluorescence quantum yield was about 5-times higher and an appreciably lower percentage of dye could be washed out with albumin-containing buffer (Fig. 1, Table 1). These observations suggest that in bilayer leaflets with less tight packing of the acyl chains many of the MC540 molecules have an orientation in which the dye is in close contact with the hydrocarbon matrix with its main axis parallel to the phospholipid molecules in the bilayer. The fluorescence characteristics

of MC540 bound to BNML cells indicate that the dye is located in the plasma membrane of these cells in a comparable way.

Flip accelerations that do not result in a significant phospholipid asymmetry, induced by treatment of cells with the SH-oxidizing agents diamide and tetrathionate [46], by heat treatment at 46° C [47] or by insertion of gramicidin into the membrane [24], did not enhance binding or produce changes of the binding characteristics (Table 1).

Changes of MC540 binding characteristics could also be observed with some other treatments, causing perturbation of the membrane structure, not related to a change in phospholipid asymmetry. Exposure of the cells to PEG 6000 or decanol did not change the amount of bound molecules but had a slight effect on the emission maximum and on the relative fluorescence quantum yield (Table 1). The effects may be attributable to the lowering of the apparent surface polarity of the cells by these compounds [26].

Heat treatment at temperatures of 48° C or higher resulted in relatively strong changes in the mode of binding, again without affecting the total amount of membrane-bound MC540. These changes can not be ascribed to a temperature-induced change of the phospholipid core to a more fluid phase, as the changed binding characteristics were observed after cooling of the cell suspension. Instead, the red-shift of the emission peak and the increased fluorescence quantum yield coincided with the development of persistent morphological changes of the cells (poikilocytosis).

Chlorpromazine perturbs the lipid structure of the erythrocyte membrane, reflected by a fluidizing effect [48] and a changed interaction between membrane lipids and intrinsic membrane proteins [49]. Treatment with this compound resulted in an enhanced amount of bound MC540, a higher emission maximum, an about 6-times higher relative fluorescence quantum yield and a decreased possibility to wash out the MC540 molecules. The enhanced amount of bound MC540 after insertion of the cationic anaesthetic chlorpromazine into the membrane may be partly caused by chlorpromazine-induced endocytosis, as indicated by the effect of temperature (Table 1). Also electrostatic interaction of MC540 with chlorpromazine is probably involved. The change in the other binding parameters again indicates a different localization and/or orientation of the dye.

The influence of an altered surface charge was studied by treatment with neuraminidase, which removes the negatively charged sialic acid residues. This treatment resulted in a slightly increased amount of MC540 bound to the cells. In line with this observation, insertion of the anionic lysophosphatidylserine ($2.4 \cdot 10^7$ molecules/cell), into the outer leaflet decreased the binding of MC540 by 24% [50]. Removal of sialic acid residues also resulted in a higher emission maximum, and a higher relative fluorescence quantum yield and thus to a change in the environment of the MC540 molecules.

Photodynamic treatment with $AlPcS_4$ and MC540 as sensitizers caused a change in the amount and in the mode of MC540 binding, which is much more pronounced in the case of $AlPcS_4$. It has been shown that treatment with $AlPcS_4$ and light causes a perturbation of the membrane structure, reflected, e.g., by enhanced transbilayer mobility of membrane lipid probes [29]. However, as discussed above, the change of MC540 binding characteristics can not be explained by increased flip-flop. Apparently photodynamic treatment induces other, as yet unknown structural changes in the erythrocyte membrane, leading to increased MC540 binding and a change of its binding characteristics.

In conclusion, some of the erythrocyte membrane perturbations evoke a change in the MC540 binding characteristics, that is qualitatively comparable to the high-affinity binding of the dye to BNML cells. However, calculated per surface area BNML cells still bind 3-times more MC540 molecules, compared to photodynamically treated red blood cells (Table 2).

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